EXHIBIT 3

Srivastava et al., "Regulation of 5-Aminolevulinate Synthase mRNA in Different Rat Tissue, "
Journal of Biological Chemistry, vol. 263, 5202-5209 (1988)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 263, No. 11, Issue of April 15, pp. 5202-5209, 1988
Printed in U.S.A.

Regulation of 5-Aminolevulinate Synthase mRNA in Different Rat Tissues*

(Received for publication, March 3, 1987)

Gopesh Srivastava, Iain A. Borthwick, Deborah J. Maguire‡, Cornelis J. Elferink‖, Michael J. Bawden, Julian F. B. Mercer§, and Brian K. May¶

From the Adelaide University Centre for Gene Technology, Department of Biochemistry, University of Adelaide, G. P. O. Box 498, Adelaide, South Australia, 5001, Australia, the ‡Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Hönggerberg, Switzerland, and the §Murdoch Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052, Australia

cDNA clones for rat liver 5-aminolevulinate synthase have been isolated and used to examine mRNA levels in different rat tissues. Northern hybridization analysis of total RNA from various rat tissues showed the presence of a single 5-aminolevulinate synthase mRNA species of estimated length 2.3 kilobases. Primer extension and RNase mapping studies indicated that the mRNA is identical in all tissues. Highest basal levels were seen in liver and heart. Administration of hemin to rats reduced the basal level of this mRNA only in liver but the heme precursor, 5-aminolevulinate (or its methyl ester), repressed the basal levels in liver, kidney, heart, testis, and brain. The drug 2-allyl-2-isopropylacetamide increased the mRNA level in liver and kidney only while human chorionic gonadotropin hormone elevated the level in testis. Administration of the heme precursor 5-aminolevulinate prevented these inductions. Nuclear transcriptional run-off experiments in liver cell nuclei showed that 2-allyl-2-isopropylacetamide and 5-aminolevulinate exert their effect by altering the rate of transcription of the 5-aminolevulinate synthase gene. The results indicate that a single 5-aminolevulinate synthase mRNA is expressed in all tissues and that its transcription is negatively regulated by heme.

5-Aminolevulinate synthase catalyzes the first step of the heme biosynthetic pathway and in the liver at least is rate-limiting (reviewed in Refs. 1 and 2). The enzyme performs a housekeeping function since all animal cells synthesize their own heme for mitochondrial cytochromes and other cellular hemoproteins. The enzyme level is normally very low in animal tissues but is greatly elevated in the liver of experimental animals following administration of a wide variety of porphyrinogenic drugs such as AIA[1] and phenobarbital (3). This biochemical response mimics the acute porphyria diseases in man where hepatic 5-aminolevulinate synthase levels are elevated during clinical attacks. Drugs which precipitate such attacks induce 5-aminolevulinate synthase levels in experimental animals. These same drugs also induce the synthesis of hepatic cytochrome P-450 proteins, which are involved in the conversion of foreign compounds to water-soluble derivatives.

Granick (3) first demonstrated that the end product heme prevented the drug-induced increase in hepatic 5-aminolevulinate synthase enzyme levels. Work in our laboratory and elsewhere (reviewed in Ref. 1) has suggested that heme acts by repressing the synthesis of 5-aminolevulinate synthase mRNA, but there has been no direct proof of this. Current evidence favors the hypothesis that the porphyrinogenic drugs act by inducing synthesis of cytochrome P-450 apoprotein which results in a reduction in the heme concentration, thus indirectly leading to an increase in 5-aminolevulinate synthase mRNA levels (1). Erythroid 5-aminolevulinate synthase is not induced by porphyrinogenic drugs (2), and this finding has led to the proposal that erythroid and hepatic 5-aminolevulinate synthases are distinct enzymes (4, 5). Indeed, it has been proposed that a multigene family exists for 5-aminolevulinate synthase with different mRNA species synthesized in different tissues (6). However, recent work suggests that 5-aminolevulinate synthase mRNA is the same in the liver and erythroid spleen of mice (7).

We are interested in determining at the molecular level how heme regulates the gene for 5-aminolevulinate synthase in liver and other tissues. cDNA (8, 9) and genomic clones (10) for chicken 5-aminolevulinate synthase have been isolated in this laboratory. In this communication we report the isolation of cDNA clones for rat liver 5-aminolevulinate synthase and provide strong evidence that the mRNA is identical in all rat tissues examined. We have established that drugs increase the level of 5-aminolevulinate synthase mRNA in a tissue-specific fashion and that heme represses levels of this mRNA in all tissues examined except erythroid spleen. Unequivocal evidence has been obtained that the altered levels of hepatic 5-aminolevulinate synthase mRNA observed after drug or heme administration are due to changes in the rate of transcription of the gene.

EXPERIMENTAL PROCEDURES

Materials—AIA was a generous gift from Roche, Australia. Hemin (ferriprotoporphyrin IX chloride) was supplied by Porphyrin Products, Logan, UT. A chicken β-actin cDNA clone in pBR322 (insert 1.8 kb) was provided by S. Dalton and a chicken serum albumin cDNA clone in pBR322 (insert 2 kb) by A. H. Hobbs. All other materials were purchased from sources previously described (9, 11).

Treatment of Animals—Male albino Wistar rats (200 g body weight) were given injections of AIA (80 mg) subcutaneously, 5-

* This research was supported in part by a grant from the National Health and Medical Research Council of Australia and by a Commonwealth Special Research Centre grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03180.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: AIA, 2-allyl-2-isopropylacetamide; SDS, sodium dodecyl sulfate; HCG, human chorionic gonadotropin hormone; RNase, ribonuclease; kb, kilobase(s); bp, base pair(s).

aminolevulinate or its methyl ester (333 mg) via the intraperitoneal cavity, or hemin (1.5 mg) via the tail vein. For induction with AIA over 12 h, a second injection of AIA was given at 7 h. 5-Aminolevulinate or hemin was given immediately after AIA injection. Rats were treated with HCG over 48 h by repeated subcutaneous injections (20 units) at 12, 24, and 36 h; 5-aminolevulinate was administered at 36 h and total mRNA isolated after a further 12 h. To obtain anemic rats, phenylhydrazine HCl (1.5 mg) was injected subcutaneously on 5 consecutive days and mRNA isolated on the sixth day. Anemic rats were treated with 5-aminolevulinate 12 h prior to death.

For developmental studies, Sprague-Dawley rats were used. Fetal ages were estimated from the time of mating; males were placed with females overnight and the following morning taken as day 0 of gestation. Total RNA was isolated essentially by the method of Liu *et al.* (12) from pooled individuals of at least 2 litters for each age.

Construction and Screening of a Rat Liver cDNA Library—Liver poly(A)$^+$ RNA from rats treated with AIA was used to construct a cDNA library. Double-stranded cDNA was synthesized by the procedure of Gubler and Hoffman (13), and DNA greater than 1600 bp was annealed with dG-tailed *Pst*I-digested pBR322 and transformed into *Escherichia coli* MC1061. Recombinant plasmids were screened with a mixture of ^{32}P nick-translated probes comprising the four *Pst*I inserts from the chicken 5-aminolevulinate synthase clone, p105B1 (9).

The clone p101B1 was sequenced by digestion of plasmid with either *Hpa*II, *Pst*I, *Sau*3A1, or *Taq*I and fragments sequenced by the method of Sanger *et al.* (14).

Analysis of RNA—Total RNA was isolated from rat tissues by the guanidine hydrochloride extraction procedure of Brooker *et al.* (15). Poly(A)$^+$ mRNA was prepared by oligo(dT)-cellulose chromatography.

For Northern blot analysis, RNA was electrophoresed in 1.0% agarose gels containing 1.1 M formaldehyde as described (11). RNA was transferred to nitrocellulose filters (BA85 from Schleicher & Schuell) and hybridized to the ^{32}P nick-translated rat 5-aminolevulinate synthase cDNA clone p101B1 (10 ng/ml) in a solution containing 50% formamide, 5 × SSPE (0.9 M NaCl, 50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA), 5 × Denhardt's (0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin), 0.1% SDS, 0.5% sodium pyrophosphate, and 200 μg/ml denatured salmon sperm DNA at 42 °C for 20 h. Filters were washed finally in 0.1 × SSPE containing 0.1% SDS at 60 °C for 40 min. For low stringency conditions filters were hybridized as above and washed in a solution containing 2 × SSPE and 0.1% SDS at 50 °C for 40 min. Molecular size markers consisted of DNA fragments generated by *Acc*I digestion of pBR322.

RNA was denatured and bound to nitrocellulose filters using either a slot or dot blot apparatus (Schleicher & Schuell). Hybridization conditions were as described above. The amounts of RNA in Northern and slot blots were quantitated using an LKB laser densitometer.

Primer extension analysis using poly(A)$^+$ RNA from different rat tissues and 5′-^{32}P-labeled synthetic primers complementary to the coding strand of p101B1 was carried out according to the method of McKnight *et al.* (16). The extended products were analyzed by electrophoresis on 8 M urea, 6% polyacrylamide gels with a dideoxy sequence ladder of M13 bacteriophage DNA as size standards or ^{32}P-labeled *Hpa*II fragments from pBR322.

RNase Mapping—The three *Pst*I restriction fragments of p101B1 (see Fig. 5) were individually subcloned into *Pst*I cut pGEM-1 vector (Promega Biotech). Two probes, A and E, contained the 5′ and 3′ *Pst*I fragments of p101B1, respectively (see Fig. 5). The plasmid containing the largest *Pst*I fragment of p101B1 was further digested with appropriate restriction enzymes to generate three subclones of suitable size for RNase mapping; restriction enzyme removal of a *Bam*HI fragment (one *Bam*HI site in polylinker) generated a clone for the protection of a 364-bp *Bam*HI-*Pst*I fragment (probe D). Digestion of the plasmid with *Bgl*II and *Hind*III (polylinker site) allowed the religation of a clone for the protection of a 631-bp *Pst*I-*Bgl*II fragment (probe B). Probe C was generated by directionally cloning the 699-bp *Bam*HI/*Sal*I fragment into a *Bam*HI/*Sal*I cut pGEM1 vector.

RNA probes uniformly labeled with [^{32}P]UTP were generated *in vitro* from the five recombinant pGEM plasmids using either T7 or SP6 polymerase as described (11). Specific activities of about 10^9 cpm/μg RNA were routinely obtained. Full-length transcripts were isolated on a 5% polyacrylamide sequencing gel and eluted in 500 mM ammonium acetate, 1 mM EDTA, 0.5% SDS for 3–6 h at 37 °C. RNase mapping using RNase A and T1 was carried out as described previously (11) and protected fragments analyzed following electrophoresis on a 5% polyacrylamide sequencing gel and autoradiography.

Nuclear Transcription Assays—Nuclei were isolated from rat liver as described by Schibler *et al.* (17). The transcription reactions contained 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1.2 mM dithiothreitol, 30% glycerol, 2 μM UTP, 1 mM each of ATP, CTP, and GTP, 100 μCi of [α-^{32}P]UTP, and 1.5 × 10^7 nuclei in a final volume of 150 μl. These were incubated at 26 °C for 15 min, and ^{32}P-labeled RNA was extracted as described by Vannice *et al.* (18). Rats were induced with AIA for 4 h. When 5-aminolevulinate was administered, it was injected 10 h prior to AIA treatment and the nuclei prepared 4 h later. For quantitation of specific transcripts, 5 μg of the appropriate cloned DNAs (double-stranded DNA from the chicken serum albumin and β-actin cDNA clones or single-stranded DNA from an M13 phage clone containing the 1.7-kb *Pst*I fragment of p101B1) were denatured and applied to a nitrocellulose filter using a slot blot apparatus. Filters were prehybridized in 1 ml of 50% formamide, 5 × SSC, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 100 μg/ml *E. coli* tRNA, and 0.2% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin at 52 °C overnight. Hybridization was carried out in the same solution with 2 × 10^6 cpm of ^{32}P-labeled RNA for 72 h at 52 °C. Filters were washed twice at room temperature for 30 min in 2 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate and then twice in 0.5 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 55 °C for 60 min. The hybridization signals were quantitated by laser densitometric scanning.

RESULTS

Isolation of Rat Liver 5-Aminolevulinate Synthase cDNA Clones—A cDNA library was constructed using poly(A)$^+$ RNA from livers of rats induced with the porphyrinogenic drug AIA. Size-selected double-stranded cDNA was used to construct a library of 4800 recombinant clones which were screened using a mixture of ^{32}P-labeled cDNA probes prepared from the four *Pst*I fragments of a previously isolated chicken liver 5-aminolevulinate synthase cDNA clone (9). Four clones gave positive hybridization signals, and the largest of these, p101B1, was sequenced. The sequence contained an open reading frame of 1929 nucleotides from nucleotides 17 to 1945 giving a predicted protein of 642 amino acids (Fig. 1). The sequence of the first 15 N-terminal amino acids of mature 5-aminolevulinate synthase purified from the mitochondria of drug-induced rat liver (19) was determined and shown to be identical to that deduced from the nucleotide sequence of p101B1 from position 185 to 229. This shows that the glutamine (at nucleotide 185) is the N-terminal amino acid of the mature protein. Upstream from this codon there are three in-frame d(ATG) codons. The d(ATG) codon at nucleotide 17 is assumed to be the initiation codon since it would result in the translation of a 5-aminolevulinate synthase precursor with a presequence of size 6 kDa in agreement with the size estimated from previous studies (19).

The deduced protein sequence of rat liver 5-aminolevulinate synthase precursor was compared with that of chicken (9) and mouse (7). The sequence of rat precursor was very similar to that of chicken, both in the N-terminal presequence (56 amino acids) and over most of the mature protein sequence. Surprisingly, the rat protein sequence showed less overall homology to the mouse enzyme, and indeed no homology existed within the presequence segments.

Northern Analysis of 5-Aminolevulinate Synthase mRNA—The size of 5-aminolevulinate synthase mRNA in different rat tissues was determined by Northern blot analysis. Total RNA was isolated from the liver, kidney, brain, and testis of untreated rats. Tissues were also examined following treatments which are known to elevate 5-aminolevulinate synthase activity levels; for this total RNA was isolated from AIA-treated rat liver and kidney, HCG-treated rat testis, and spleen of rats rendered anemic by phenylhydrazine treatment.

The RNA samples were fractionated on a formaldehyde

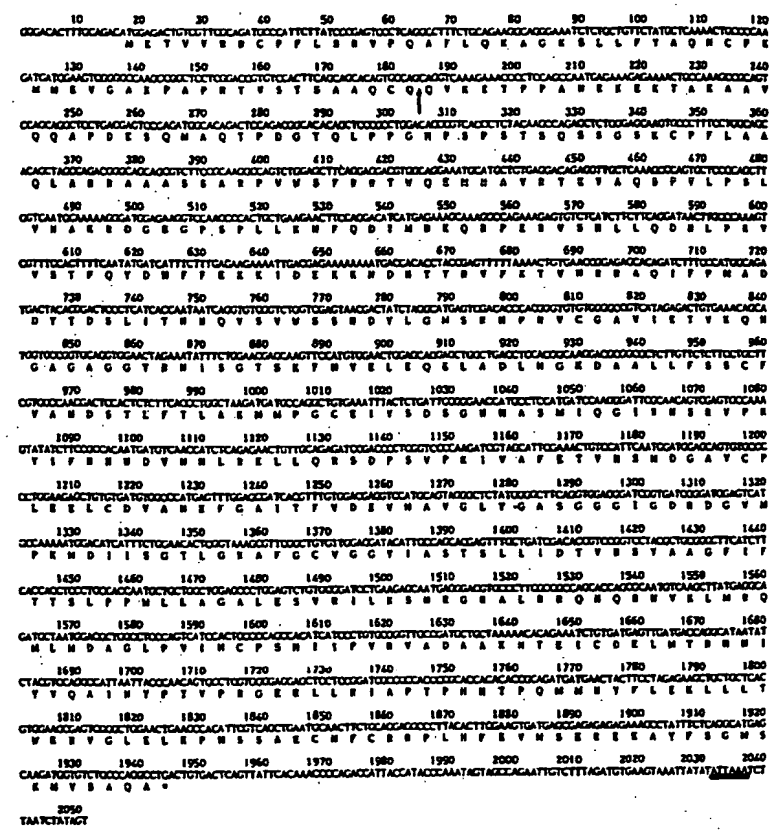


FIG. 1. **Nucleotide and predicted amino acid sequence of rat liver 5-aminolevulinate synthase.** The clone has 5′- and 3′-noncoding regions of 16 and 112 nucleotides, respectively. A possible polyadenylation signal ATTAAA is *underlined*, and the termination codon is *asterisked*. The *arrow* indicates the cleavage site of the presequence.

agarose-denaturing gel and probed with ^{32}P-labeled p101B1. In all tissues, only a single 5-aminolevulinate synthase mRNA species was detected, estimated to be 2.3 kb in length (Fig. 2). In some tracks, very faint extra bands were visible and were probably due to ribosomal RNA. The results therefore show that there is no difference in size between 5-aminolevulinate synthase mRNA from either untreated or induced rat tissues.

It was also of interest to determine the 5-aminolevulinate synthase mRNA during fetal development. A single 5-aminolevulinate synthase mRNA species (2.3 kb in length) was detected at day 14 of gestation when the liver is largely erythroid in nature (20) through to the adult stage (Fig. 3). The level of mRNA peaked prior to birth at gestation days 20 and 21. This level then declined significantly at birth and in the adult had returned to the level seen prior to birth.

Primer Extension and RNase Mapping Studies of 5-Aminolevulinate Synthase mRNA from Rat Tissues—Further studies were carried out to determine whether 5-aminolevulinate synthase mRNA was identical in different tissues of rat. Of particular interest was the relationship of the nonerythroid to erythroid mRNA in view of the proposal that these mRNAs are distinct in the chicken (6). A 5′-γ-^{32}P-labeled 23-nucleotide oligomer complementary to the coding sequence of p101B1 from nucleotides 17 to 40 (Fig. 1) was used in a primer extension reaction on poly(A)$^+$ RNA isolated from tissues of untreated and treated rats (see legend to Fig. 4). With all mRNA samples, two bands, 144 and 147 nucleotides in length, were seen following electrophoresis of the extension products and detection by autoradiography (Fig. 4). Such doublets have been observed previously and may be due to an artifact of the reverse transcriptase reaction (21) or to initiation of mRNA synthesis at an alternative nucleotide. A second set of primer extension reactions was performed using a primer complementary to a sequence in the 5′-untranslated region of p101B1 from nucleotides 3 to 26. The rationale for this was that different mRNAs in different tissues may be homologous in their coding sequences but different in the 5′-untranslated region. With all tissues a doublet was observed with bands 108 and 111 nucleotides in length (result not shown). The results suggest that the 5′-untranslated region of the 5-aminolevulinate synthase mRNA from different tissues is the same.

As indicated earlier, the 5′-untranslated region of p101B1 is 16 nucleotides in length. The primer extension analysis indicates that in the mRNA, this region is 100 or 103 nucleotides in length, and p101B1 is, therefore, 84 or 87 nucleotides short of being full-length.

For RNase mapping experiments, five [α-^{32}P]UTP-labeled RNA probes complementary to rat hepatic 5-aminolevulinate synthase mRNA were synthesized by *in vitro* transcription of pGEM-1 plasmids containing appropriate restriction fragments of p101B1 (see Fig. 5). The probe sequences (*A–E*)

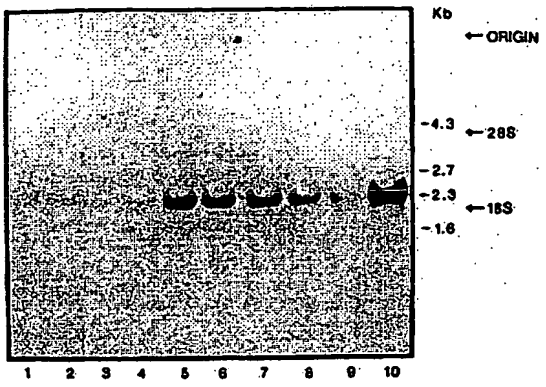


FIG. 2. Northern blot analysis of 5-aminolevulinate synthase mRNA in rat tissues. Total RNA from tissues of untreated or treated rats was electrophoresed on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. Molecular size markers consisted of DNA fragments generated by *Acc*I digestion of pBR322. The positions of rRNA markers are shown. *Lane 1*, erythroid spleen (50 μg); *lane 2*, brain (50 μg); *lane 3*, testis (20 μg); *lane 4*, testis (HCG-treated rat) (10 μg); *lane 5*, heart (20 μg); *lane 6*, heart (AIA-treated rat) (20 μg); *lane 7*, kidney (AIA-treated rat) (20 μg); *lane 8*, kidney (50 μg); *lane 9*, liver (5 μg); *lane 10*, liver (AIA-treated rat) (5 μg).

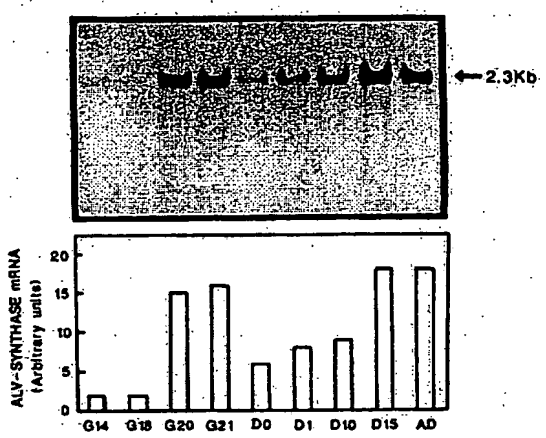


FIG. 3. Developmental profile of 5-aminolevulinate (*ALV*) synthase mRNA. Total RNA (10 μg) from rat liver at various stages of development was resolved on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. The size of the mRNA was estimated as in Fig. 2, and mRNA levels were quantitated from the Northern blot by densitometric scanning and shown as *bar graphs*. Loadings of RNA were shown to be uniform by ethidium bromide staining of the gel. *G*, gestation day; *D*, day following birth; *AD*, adult, 12 weeks.

complementary to the mRNA were 73, 631, 699, 364, and 210 nucleotides in length, respectively, and spanned the entire mRNA except for 83 nucleotides not present in the cDNA clone at the extreme 5′-end and 90 nucleotides between the *Bgl*II and *Sal*I sites (see Fig. 5). Poly(A)$^+$ RNA samples were hybridized to these probes, and nonhybridized RNA was digested with RNase A and T1. The protected radiolabeled fragments were resolved on a 5% polyacrylamide sequencing gel.

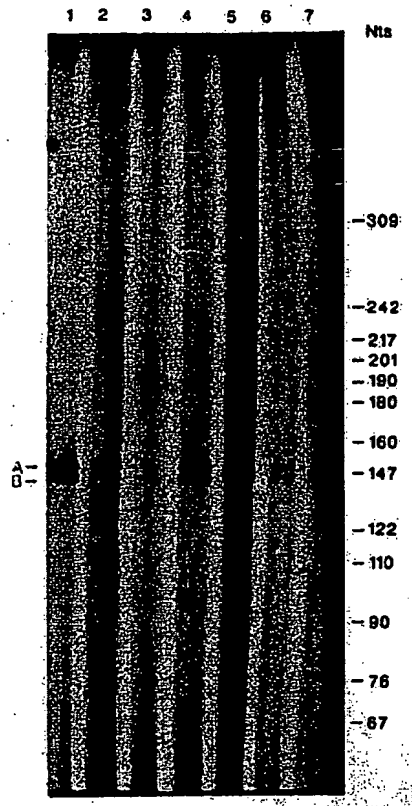


FIG. 4. Primer extension analysis of 5-aminolevulinate synthase mRNA in various rat tissues. A chemically synthesized 23-nucleotide oligomer was 5′-phosphorylated with [γ-^{32}P]ATP and used to primer extend on poly(A)$^+$ RNA from untreated rat liver (10 μg) (*lane 2*); brain (50 μg) (*lane 3*); heart (10 μg) (*lane 4*); erythroid spleen (50 μg) (*lane 7*); AIA-treated rat liver (5 μg) (*lane 1*); kidney (10 μg) (*lane 5*); HCG-treated rat testis (10 μg) (*lane 6*). Products were analyzed on an 8 M urea, 6% polyacrylamide gel with ^{32}P-labeled *Hpa*II fragments from pBR322 as size standards. *Bands A* and *B* are 147 and 144 nucleotides in length, respectively. *Nts*, nucleotides.

For liver and erythroid spleen mRNA, all five RNA probes were employed. Fragments representing full-length protection products for each probe were observed, establishing that these mRNAs are very likely identical. Mapping with probe E which spans the 3′-noncoding end of the liver mRNA revealed two bands smaller than the expected full-length product of 210 nucleotides (Fig. 5). These additional bands may be caused by heterogeneity in the lengths of the poly(A) tails of isolated liver mRNA. In other experiments only probe B was used with mRNA from liver, kidney, brain, heart and testis of untreated rats, from kidney of AIA-treated rats, and from testis of HCG-treated rats. This probe fully protected the expected 631-nucleotide fragment in all the mRNA samples examined (results not shown). In summary, this study, together with Northern blot and primer extension analyses, provides compelling evidence that 5-aminolevulinate synthase mRNA is identical in all rat tissues.

Measurement of Basal Levels of 5-Aminolevulinate Synthase mRNA in Rat Tissues—Total RNA was isolated from tissues of untreated rats, and 5-aminolevulinate synthase mRNA

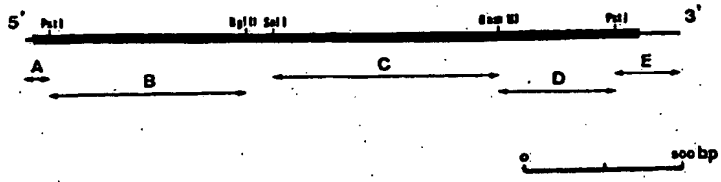
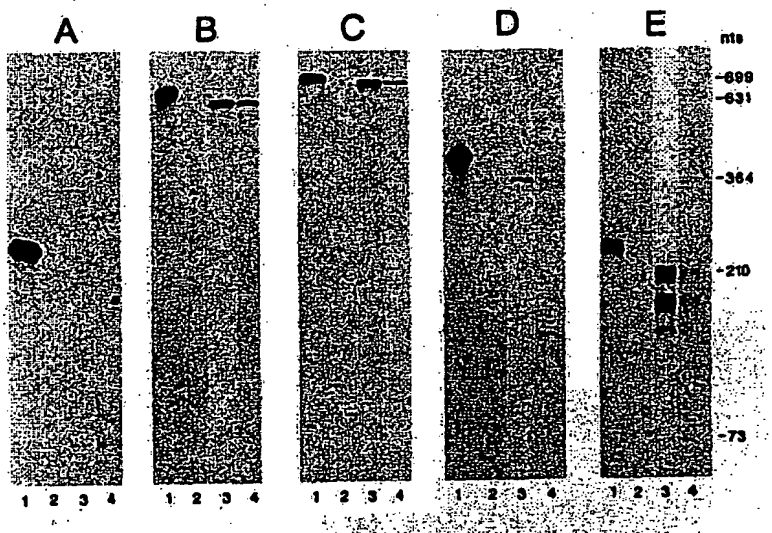


FIG. 5. RNase mapping of rat liver and erythroid spleen 5-aminolevulinate synthase mRNA. The cDNA clone, p101B1, is represented in the upper part of the figure with the coding and noncoding regions depicted as *heavy* and *light lines*, respectively. The *double-headed arrows* below show the relationship of the RNA probes *A–E* to p101B1. ^{32}P-Labeled RNA probes were hybridized to 2.5 μg of poly(A)$^+$ RNA from AIA-induced liver (*lane 3*) or erythroid spleen (*lane 4*) and incubated with RNase A and T1. Probes incubated in the absence of poly(A)$^+$ RNA were either untreated (*lane 1*) or treated (*lane 2*) with RNase A and T1. Protected fragments were analyzed by electrophoresis and autoradiography as described under "Experimental Procedures." The numbers displayed to the right of *panel E* correspond to the expected nucleotide lengths of the protected fragments. *nts*, nucleotides.



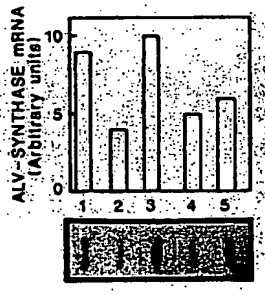
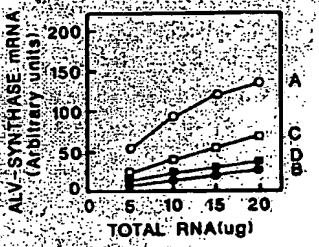


FIG. 6. Measurement of basal 5-aminolevulinate (*ALV*) synthase mRNA levels in rat tissues. Total RNA (10 μg) from tissues of untreated rats was applied as slots on nitrocellulose and hybridized to the nick-translated *PstI* inserts of p101B1. 5-Aminolevulinate synthase mRNA levels were quantitated by densitometric scanning and shown as *bar graphs*. *Lane 1*, liver; *lane 2*, kidney; *lane 3*, heart; *lane 4*, brain; *lane 5*, testis.



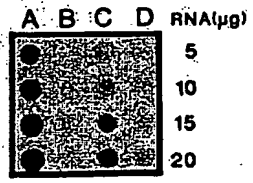


FIG. 7. Effect of hemin on 5-aminolevulinate (*ALV*) synthase mRNA levels in rat liver. Total RNA was isolated from rat liver 2 h after administration of AIA, hemin, or both. Amounts of total RNA (5–20 μg) were applied as dots on nitrocellulose and hybridized to the nick-translated *PstI* inserts of p101B1. mRNA levels were quantitated by densitometric scanning. *Lane A*, AIA treated; *lane B*, AIA and hemin treated; *lane C*, untreated; *lane D*, hemin treated.

amounts were quantitated. As can be seen in Fig. 6, the heart has the highest level of 5-aminolevulinate synthase mRNA, slightly above that of the liver, while in kidney, brain, and testis there is approximately half this level.

Effect of Hemin and 5-Aminolevulinate on 5-Aminolevulinate Synthase mRNA Levels in Untreated and Drug-treated Rats—The effect of hemin on liver 5-aminolevulinate synthase mRNA levels was first examined. Fig. 7 shows that

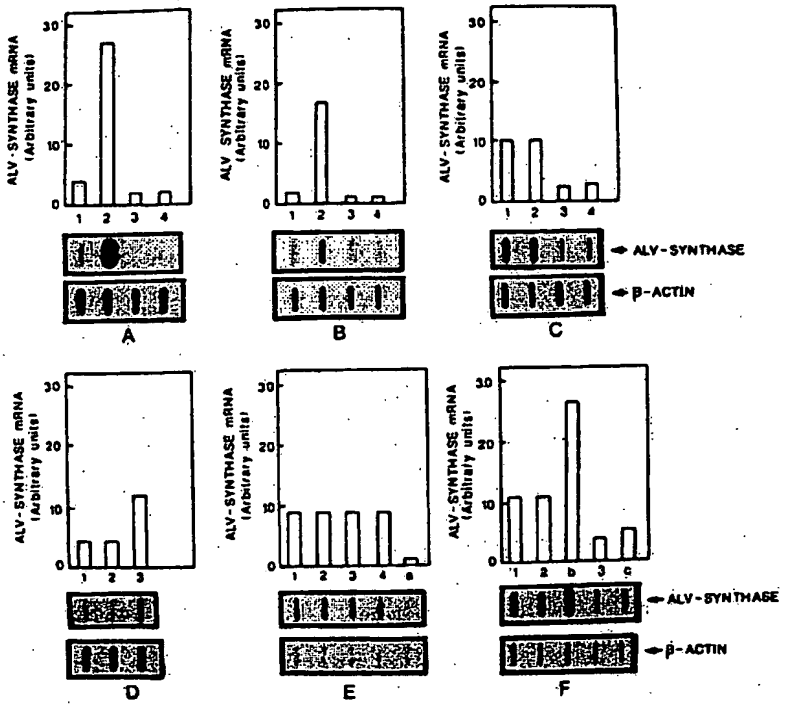


FIG. 8. Measurement of 5-aminolevulinate (*ALV*) synthase mRNA levels in rat tissues after 5-aminolevulinate treatment. Total RNA (10 μg) from tissues 12 h after administration of AIA or 5-aminolevulinate and from testis after HCG administration was applied as slots on nitrocellulose and hybridized to the nick-translated *Pst*I inserts of p101B1. mRNA levels were quantitated by densitometric scanning and shown as bar graphs. *Panel A*, liver; *panel B*, kidney; *panel C*, heart; *panel D*, erythroid spleen; *panel E*, brain; *panel F*, testis. *Lane 1*, untreated; *lane 2*, AIA treated; *lane 3*, 5-aminolevulinate treated; *lane 4*, AIA and 5-aminolevulinate treated; *lane a*, 5-aminolevulinate-methyl ester treated; *lane b*, HCG treated; *lane c*, HCG and 5-aminolevulinate treated.

treatment of rats with the drug AIA over a 2-h period increased the level of 5-aminolevulinate synthase mRNA (Fig. 7, *A* and *C*). Administration of hemin either to drug-treated or untreated rats reduced the level of 5-aminolevulinate synthase mRNA to below that of basal levels (Fig. 7, *B* and *D*).

In similar studies it was found that hemin had no effect on the mRNA level in the extrahepatic tissues investigated (data not shown). It was possible, however, that injected hemin was not reaching or not entering the cells of these tissues. Anderson *et al.* (22) has reported that administered 5-aminolevulinate is taken up by many tissues and converted rapidly to heme. The effect of 5-aminolevulinate on 5-aminolevulinate synthase mRNA levels in different rat tissues was, therefore, studied in both normal and drug-treated animals. Of the tissues studied only liver and kidney showed induction by AIA; 5-aminolevulinate administration completely prevented the increase in mRNA levels in both tissues (Fig. 8, *panels A* and *B*). Similarly, basal levels of 5-aminolevulinate synthase mRNA in the liver, kidney, heart, and testis were reduced by 5-aminolevulinate to low levels (Fig. 8). In the case of brain, 5-aminolevulinate had no effect, but the methyl ester derivative of 5-aminolevulinate significantly reduced the mRNA level (Fig. 8, *panel E*) presumably because this compound can readily cross the blood-brain barrier. Erythroid spleen was an exception with 5-aminolevulinate treatment resulting in an increase in 5-aminolevulinate synthase mRNA levels (Fig. 8, *panel D*).

In other experiments, it was shown that 5-aminolevulinate prevented the HCG induction of 5-aminolevulinate synthase mRNA in the testis (Fig. 8, *panel F*).

In all the experiments described here the level of β-actin mRNA in the tissues was quantitated (see Fig. 8, but not shown graphically) and was found to be essentially unchanged indicating that the response of 5-aminolevulinate synthase mRNA levels to AIA, HCG, or 5-aminolevulinate did not reflect a general cellular event. We also measured 5-aminolevulinate synthase activity in homogenates of all tissues examined above, and the amounts detected correlated closely with the changes observed in 5-aminolevulinate synthase mRNA levels (results not shown).

Hemin Acts at the Transcriptional Level in the Liver—The experiments described above show that in different tissues AIA and heme regulate the levels of 5-aminolevulinate synthase mRNA. We investigated whether, in liver, this reflected transcriptional control. Rat livers were removed, nuclei isolated, and gene transcription activities quantitated. In this *in vitro* system, it was established that incorporation by the nuclei of [α-^{32}P]UTP into total RNA was linear for at least 30 min, and the extent of incorporation was similar in nuclei from untreated rats and rats treated with AIA, 5-aminolevulinate, or both. Additionally, α-amanitin (2 μg/ml) in the reaction mixture inhibited total RNA synthesis by about 40% and completely inhibited the synthesis of specific transcripts.

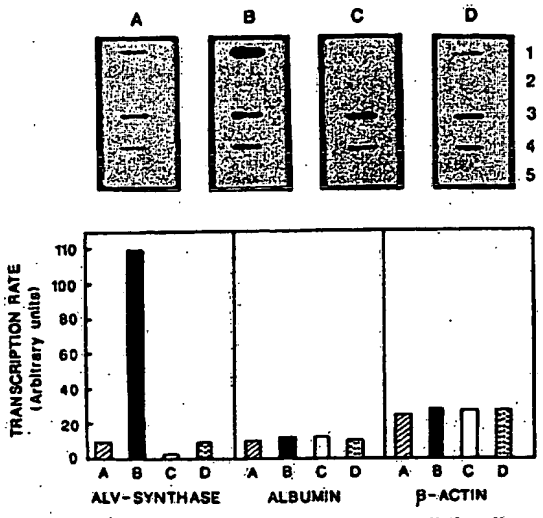


FIG. 9. Effect of 5-aminolevulinate on 5-aminolevulinate (*ALV*) synthase gene transcription. Rats were treated with AIA for 4 h. 5-Aminolevulinate was administered 10 h prior to AIA, and [^{32}P]RNA subsequently was isolated from rat liver nuclei and hybridized to nitrocellulose filter-bound cloned DNA. Transcription rates were quantitated from the slot blots by densitometric scanning and densities in arbitrary units shown as *bar graphs* after correction for the appropriate vector background controls. *A*, untreated rats; *B*, AIA treated; *C*, 5-aminolevulinate treated; *D*, AIA and 5-aminolevulinate treated. DNA clones: *lane 1*, 5-aminolevulinate synthase; *lane 2*, M13mp19; *lane 3*, chicken β-actin; *lane 4*, chicken serum albumin; *lane 5*, pBR322.

Our results show that administration of AIA alone for 12 h resulted in a 10-fold increase in the transcriptional rate of the 5-aminolevulinate synthase gene (see Fig. 9). This correlated with a 7-fold increase in hepatic 5-aminolevulinate synthase mRNA levels measured at this time (see Fig. 8, *panel A*).

The effect of 5-aminolevulinate on the transcription rate of the 5-aminolevulinate synthase gene was investigated. Administration of this compound to untreated rats significantly reduced the low basal transcriptional rate of the 5-aminolevulinate synthase gene (Fig. 9). Administration of 5-aminolevulinate to AIA-treated rats prevented the drug-induced increase in the rate of gene transcription (Fig. 9). A corresponding reduction in the level of hepatic 5-aminolevulinate synthase mRNA was observed (Fig. 8, *panel A*). Throughout this work, the transcriptional rate of the serum albumin gene measured as a control was unchanged although that of the β-actin gene was slightly elevated by either AIA or 5-aminolevulinate treatment (Fig. 9). The reason for the latter is unknown. Other control experiments established that the presence of 5-aminolevulinate (50 μM) or hemin (0.01–10 μM) in the transcription reaction had no effect on any of the genes under test. Overall, these results demonstrate that in the liver, AIA and 5-aminolevulinate treatment modulates 5-aminolevulinate synthase mRNA levels by altering the rate of gene transcription.

DISCUSSION

The early work of Granick (3) established that in chick embryos drug inducibility of hepatic 5-aminolevulinate synthase activity is prevented by the simultaneous administration of heme. The mechanism of this has remained a central question ever since. It was originally postulated that heme and drugs compete for a site on a gene-controlling protein. This was rendered improbable by the work of Srivastava *et al.* (23) who showed that heme repression appeared to be the sole control and that drug induction was probably a secondary consequence of heme removal (reviewed in Ref. 1). Since the original postulate, it has been variously claimed that heme works at the translational and transcriptional level, and considerable confusion has existed (1). This was, in part, due to studies being done at the enzymic level and to the complication that hemin prevents entry of newly synthesized 5-aminolevulinate synthase into the mitochondrion (1).

In this work we have studied 5-aminolevulinate synthase control at the mRNA level. To this end a cDNA clone for rat liver 5-aminolevulinate synthase has been isolated and sequenced. Using probes derived from this, control of 5-aminolevulinate synthase mRNA in various rat tissues has been examined.

The first question investigated was whether there exists in rat a single mRNA for 5-aminolevulinate synthase or a multiplicity of them. This question arises from the suggestion that a family of 5-aminolevulinate synthase genes exists in chicken (6). The results here give compelling evidence that the 5-aminolevulinate synthase mRNA in all rat tissues examined is the same and that only a single species exists. This is in keeping with our recent conclusion that the erythroid 5-aminolevulinate synthase in chicken is coded for by the same gene as that in the liver (11). The work also shows that the rat liver 5-aminolevulinate synthase mRNA present during fetal development is indistinguishable on Northern blots from the adult form.

Although drug induction of 5-aminolevulinate synthase in liver is well known, the inducibility in other tissues has not been well documented. We show here that 5-aminolevulinate synthase mRNA is induced by AIA only in the liver and kidney of rat. Correlating with this, the level of the phenobarbital-inducible cytochrome P-450 b/e mRNAs are elevated by AIA specifically in these tissues.[1] Interestingly, 5-aminolevulinate synthase mRNA in testis is induced by HCG which also induces tissue-specific cytochrome P-450 proteins (24). These results support the proposal (1) that inducibility of 5-aminolevulinate synthase is a secondary consequence of heme depletion due to induction of cytochrome P-450 apoprotein which takes up heme as a prosthetic group.

A basal level of 5-aminolevulinate synthase mRNA was detected in all rat tissues examined. A question which has not been addressed previously is whether heme controls the basal level of 5-aminolevulinate synthase mRNA or only the drug-stimulated increase and also whether this heme control is confined to liver. The results in this paper establish that heme repression of 5-aminolevulinate synthase mRNA levels occurs in all rat tissues studied, with the exception of erythroid spleen, with both basal and induced levels being affected. In erythroid spleen the level of 5-aminolevulinate synthase mRNA was elevated. Possibly this is indirectly due to the induction of heme oxygenase (25). A reservation in this work is that possibly for cell permeability reasons, administered hemin affects only liver 5-aminolevulinate synthase mRNA levels. However, administered 5-aminolevulinate (or its methyl ester in the case of brain) lowers the 5-aminolevulinate synthase mRNA level in all tissues except spleen. It seems most probable that repression by heme is being observed since there is no evidence that 5-aminolevulinate itself has a regulatory role, and it is known that injected 5-aminolevulinate is rapidly converted to heme in many tissues (22).

[1] G. Srivastava, unpublished data.

A final question concerns the level at which heme control is exerted. We have conclusively established that in liver, heme regulates 5-aminolevulinate synthase mRNA levels by acting predominantly, if not exclusively, to inhibit transcription of the 5-aminolevulinate synthase gene.

This work places the 5-aminolevulinate synthase gene in a small group of animal genes (26, 27) known to be negatively controlled by a metabolic end product. The molecular basis for the regulation of the 5-aminolevulinate synthase gene is an important problem to be investigated.

Acknowledgments—We would like to thank W. H. Elliott for valuable discussions throughout the course of the work and for critically reading the manuscript. The excellent technical assistance of M. Cresp is gratefully acknowledged.

REFERENCES

1. May, B. K., Borthwick, I. A., Srivastava, G., Pirola, B. A., and Elliott, W. H. (1986) *Curr. Top. Cell. Regul.* **28**, 233–262
2. Kappas, S., Sassa, S., and Anderson, K. R. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngarden, J. B., Frederickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th Ed., pp. 1301–1384, McGraw-Hill Publications, Minneapolis, MN
3. Granick, S. (1966) *J. Biol. Chem.* **241**, 1359–1375
4. Bishop, D. F., Kitchen, H., and Wood, W. A. (1981) *Arch. Biochem. Biophys.* **206**, 380–391
5. Watanabe, N., Hayashi, H., and Kikuchi, G. (1983) *Biochem. Biophys. Res. Commun.* **113**, 377–383
6. Yamamoto, M., Yew, N. S., Federspiel, M., Dodgson, J. B., Hayashi, N., and Engel, J. D. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3702–3706
7. Schoenhaut, D. S., and Curtis, P. J. (1986) *Gene (Amst.)* **48**, 55–63
8. Borthwick, I. A., Srivastava, G., Hobbs, A. A., Pirola, B. A., Brooker, J. D., May, B. K., and Elliott, W. H. (1984) *Eur. J. Biochem.* **144**, 95–99
9. Borthwick, I. A., Srivastava, G., Day, A. R., Pirola, B. A., Snoswell, M. A., May, B. K., and Elliott, W. H. (1985) *Eur. J. Biochem.* **150**, 481–484
10. Maguire, D. J., Day, A. R., Borthwick, I. A., Srivastava, G., Wigley, P. L., May, B. K., and Elliott, W. H. (1986) *Nucleic Acids Res.* **14**, 1379–1391
11. Elferink, C. J., Srivastava, G., Maguire, D. J., Borthwick, I. A., May, B. K., and Elliott, W. H. (1987) *J. Biol. Chem.* **262**, 3988–3992
12. Liu, C. P., Slate, O. L., Gravel, R., and Ruddle, F. H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4503–4506
13. Gubler, U., and Hoffman, B. J. (1983) *Gene (Amst.)* **25**, 263–269
14. Sanger, F., Nicklen, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
15. Brooker, J. D., May, B. K., and Elliott, W. H. (1980) *Eur. J. Biochem.* **106**, 17–24
16. McKnight, S. L., Gavis, E. R., and Kingsbury, R. (1981) *Cell* **25**, 385–398
17. Schibler, U., Hagenbuchle, O., Wellauer, P. K., and Pittet, A. C. (1983) *Cell* **36**, 501–508
18. Vannice, J. C., Taylor, J. M., and Ringold, G. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4241–4245
19. Srivastava, G., Borthwick, I. A., Brooker, J. D., May, B. K., and Elliott, W. H. (1982) *Biochem. Biophys. Res. Commun.* **109**, 305–312
20. Greengard, O., Federman, N., and Knox, W. E. (1972) *J. Cell Biol.* **52**, 2361–2372
21. Luse, D. R., Haynes, J. R., van Leeuwen, D. Schon, E. A., Cleary, M. L., Shapiro, S. G., Lingrel, J. B., and Roeder, R. G. (1981) *Nucleic Acids Res.* **9**, 4339–4354
22. Anderson, K. E., Drummond, G. S., Freddara, V., Sardana, M. K., and Sassa, S. (1981) *Biochim. Biophys. Acta* **676**, 289–299
23. Srivastava, G., Brooker, J. D., May, B. K., and Elliott, W. H. (1980) *Biochem. J.* **188**, 781–788
24. John, M. E., John, M. C., Simpson, E. R., and Waterman, M. R. (1985) *J. Biol. Chem.* **260**, 5760–5767
25. Maines, M. D. (1984) *CRC Crit. Rev. Toxicol.* **12**, 241–314
26. Osborne, T. F., Goldstein, J. L., and Brown, M. S. (1985) *Cell* **42**, 203–212
27. Boyce, F. M., Anderson, G. M., Rusk, C. D., and Freytag, S. O. (1986) *Mol. Cell. Biol.* **6**, 1244–1252